International Journal of Innovation and Applied Studies
ISSN 2028-9324 Vol. 13 No. 3 Nov. 2015, pp. 518-526

http://www.ijias.issr-journals.org/

Total phenolic compounds and antioxidant potential of quince (*Cydonia oblonga* Miller) leaf methanol extract

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ABSTRACT: Natural products continue to play a significant role in drug discovery and development processes, and many plants have already provided valuable clues for potentially bioactive compounds. Additionally, it has become evident that significant health risks and benefits are associated with dietary food choice. Studies conducted in the past few years have demonstrated that quince (*Cydonia oblonga* Miller) is a good, safe, and low-cost natural source of different classes of interesting metabolites. In particular, phenolic compounds have application as preventive or therapeutic agents in diseases in which free radicals are involved. Despite the fact that quince fresh or processed fruit properties have attracted most attention, this study focused on antioxidant activities of phenolic compounds extracted from leaves of the Tunisian local variety. Accordingly, chromatographic characterization of methanol extract revealed the existence of nine compounds corresponding to phenolic acids and flavonoids. However, only six compounds could be systematically identified namely, 4-*O*-caffeoylquinic acid, quercetin-3-*O*-rutinoside, quercetin-3-*O*-galactoside, kaempferol-3-*O*-rutinoside, kaempferol-3-*O*-glycoside, and kaempferol-3-*O*-glucoside. Total phenolic compounds and flavonoids showed respectable content as compared to common medicinal plants: 52.5 mg/g dw and 4 mg/g dw, respectively. Consequently, DPPH free radical-scavenging activity and the inhibition of pre-formed radical $ABTS^{\cdot}$+ by leaf extract were comparable to or even higher than the effect of synthetic antioxidant BHT. In addition, both tests were very highly correlated (r = 0.82) under the effect of quince leaf extract testifying to the sufficiency of only one test to highlight antioxidant potential.

KEYWORDS: quince leaves, methanol extract, phenolic compounds; antioxidant activity.

1 INTRODUCTION

Quince (*Cydonia oblonga* Miller) is a fruit tree of the Rosaceae family, whose cultivation has rapidly spread from Asia to central and southern parts of Europe, North Africa, Oceania and Americas. In Tunisia, quince has been historically grown mostly in the northern part of the country with current production estimated to 3000 t/year over 700 ha of croplands [1]. Fresh or processed quince fruits have been widely recognized as a valuable and cheap dietary source of health-promoting compounds, due to biologically active constituents which are characterized by their antioxidant, antimicrobial, anti-inflammatory, anti-proliferative, and anti-ulcerative properties [2], [3], [4], [5], [6], [7]. There has been, nonetheless, an increasing attention to the phenolic content, composition and reactivity of quince non-edible aerial parts namely, leaves and seeds [2], [8].

Quince leaves have been also historically used, after decoction or infusion, in traditional medicine for their sedative, antipyretic, anti-diarrheic and antitussive properties and for the treatment of various skin diseases [8], [9], [10], [11]. Several therapeutically focused studies showed that quince leaf extract improved antioxidant activities in the liver of hyperlipidaemia rats [11], significantly protected the human erythrocyte membrane from hemolysis [12], prevented hematotoxic stress

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induced by UVA in catfish [13], and inhibited the proliferation of human colon cancer cells (Caco-2) in a concentration-dependent manner [5].

All these health-promoting properties have been attributed to the high level of phenolic compounds contained in fruits and leaves of *Cydonia oblonga*. In general, more than 4000 phenolic compounds (phenolic acids, flavonoids or tannins) have been found at different concentration in vascular plant tissues, where they are able to act as antioxidants to prevent free radical damage to proteins, carbohydrates, lipids and DNA caused by abiotic stress [3], [12], [14], [15]. Aside to their physiological roles in plants, some of them are also favorable to human health, since they are able to act as antioxidants in a number of ways: as reducing agents, hydrogen donors, free radicals scavengers, singlet oxygen quenchers, metals chelators, enzyme inhibitors and in cell signaling, and, therefore, as cell saviors [3], [12]. Thus, scientific interest in antioxidants isolated from plant materials has been encouraged in order to ascertain whether the course of oxidative stress-related diseases can be altered.

Among the several *Cydonia oblonga* vegetal tissues, leaf characterization is relatively recent and is still understudied as compared to comestible parts such as fresh or processed fruits [3], [7], [16]. This work is among the first studies that aim to characterize phenolic compounds in Tunisian quince leaves as well as their antioxidant potential as compared to synthetic bioactive standards.

2 Materials And Methods

2.1 Leaf Sample Preparation

Fresh leaves of the local quince variety (*Cydonia oblonga* Miller) were sampled randomly from six various orchards located in the north-eastern part of Tunisia prior to leaf abscission (November). This agricultural area is famous for growing fruit trees, where quince fruits are produced to be sold fresh or mainly for jam production. At the laboratory, healthy green leaves were selected, thoroughly rinsed under running tap water then dried at 40 °C for 5 d. Dried leaves were ground until reaching a fine powder consistency then stored at 4 °C prior to analysis.

2.2 Phenolic Compound Analysis

Prior to the extraction of phenolic compounds, vegetable fats were removed from powdered leaves in a clean-up step. Accordingly, a prepared leaf sample (30 g) was extracted with n-hexane (200 mL) for 24 h in a Soxhlet apparatus. Then, the same leaf sample was air-dried again and phenolic compounds extracted with methanol (200 mL) under the same experimental conditions. The solvent extract was filtered and evaporated to dryness at 40 °C in a rotary evaporator (Heidolph Laborota 4001, Germany). Finally, residual dry extracts were re-dissolved in methanol at 1 mg/mL prior to analysis.

Phenolic compounds extracted from quince leaves were identified using high-performance liquid chromatography (Agilent 1100, USA) with diode-array detector (HPLC–DAD) coupled on line to a tandem mass spectrometer (MS/MS) (Waters Micromass Quattro Ultima, USA). Oven temperature was kept constant at 30 °C and UV spectrum acquisition was operated between 250 and 550 nm.

2.3 Total Phenol And Flavonoid Concentration

The concentration of total phenolic compounds extracted from quince leaves was measured using Folin-Ciocalteu reagent. From the dry extract solution at 1 mg/mL, 0.5 mL was mixed with 5 mL of a 1/10 ultra pure water diluted Folin-Ciocalteu reagent and 4 mL Na_2CO_3 (1 M). The mixture was shaken for 15 min at room temperature in darkness before absorbance determination at 765 nm using a UV-vis spectrophotometer (Jasco V350, Japan). Total phenolic content is determined with respect to a reference compound, gallic acid (GA), and results are expressed as mg GA equivalent per g dry weight (mg GAE/g dw) [17].

Total flavonoid content was determined by mixing 0.5 mL of dry extract (1 mg/mL) with 1.5 mL methanol, 0.1 mL $AlCl_3$, $6H_2O$ (10%), 0.1 mL $C_2H_3NaO_2$ (1 M) and 2.8 mL of ultra pure water [18]. The mixture was shaken vigorously then left to react for 30 min in darkness. Absorbance was determined at 430 nm and results expressed as mg quercetin equivalent per g dry weight (mg QE/g dw).

2.4 ANTIOXIDANT POTENTIAL OF LEAF EXTRACT

The antioxidant activity of leaf methanol extract was measured using colorimetric methods and compared to the reactivity of butylated hydroxytoluene (BHT), a synthetic antioxidant used as a food additive in small amounts [19]. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), a water-soluble analog of vitamin E, was also used to estimate and compare the antioxidant activities of quince leaves and BHT by defining their relative TEAC (Trolox Equivalent Antioxidant Capacity) expressed as mmol TEAC/g extract [20].

In this study, the antioxidant potential of quince leaf extract was evaluated using two distinct decolorization assays: 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity and reduction of pre-formed radical monocation 2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS·+). DPPH radical-scavenging activity was determined by [21] with a slight modification. Extract (1 mL) was added at increasing concentrations to 2 mL of DPPH (0.1 mM) in ethanol. The mixture was mixed vigorously and allowed to stand at room temperature in the dark for 1 h. The absorbance of the resulting solution was measured spectrophotometrically at 515 nm. A blank solution was prepared using a mixture of methanol (1 mL) and DPPH (2 mL). In parallel, BHT antioxidant activity was estimated using the same experimental protocol and Trolox standard curve was prepared in the range of 0-100 µmol (Fig. 1).

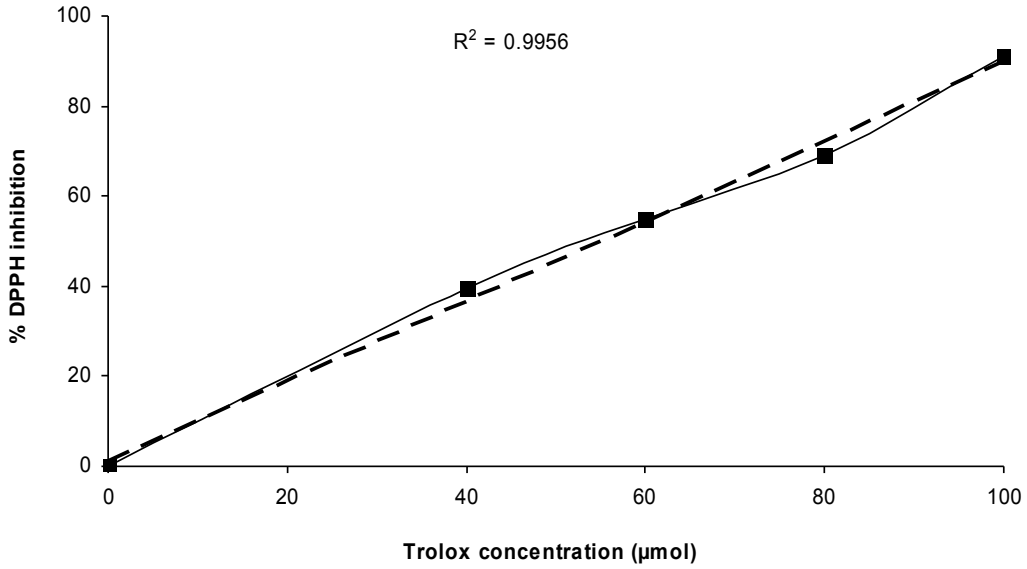

Fig. 1. Linear regression of DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity of Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid).

The free radical scavenging activity was determined by ABTS (2,2-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid) radical cation (ABTS·+) decolorization assay [22]. ABTS (2 mL) was first dissolved in ultra pure water to 7 mmol concentration. Then ABTS radical cation (ABTS·+) was produced by reacting ABTS stock solution with 2.45 mmol potassium persulfate ($K_2S_2O_8$) and kept in the dark at room temperature (20 ± 2 °C) for 12-16 h before reaction. The radical is stable in this form for more than 2 d when stored in the dark at room temperature. Before analysis the ABTS·+ solution was diluted in methanol to an absorbance of 0.7 at 734 nm. Aliquots of 150 µL of leaf sample extract were added at increasing concentrations to 2580 µL of diluted ABTS·+ solution and absorbance was read exactly 15 min after initial mixing in the dark. As for DPPH assay, BHT antioxidant activity was estimated using the same experimental protocol and Trolox standard curve was prepared in the range of 0-300 µmol (Fig. 2).

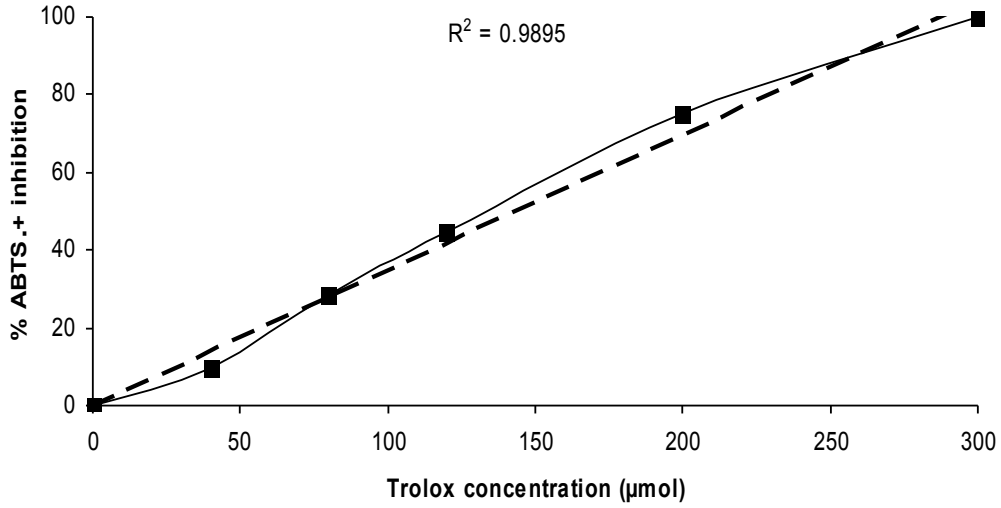

Fig. 2. Linear regression of ABTS·+ (2,2-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid) radical cation scavenging activity of Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid).

2.5 STATISTICAL ANALYSIS

All measurements were carried out in triplicate and data processed using STATISTICA 5.0 software (StatSoft Inc., USA). For both antioxidant assays, half maximal 50% inhibitory concentrations (IC_{50}) of quince leaf extract and BHT were calculated using Origin 6.0 (Microcal Software Inc., USA). ANOVA with Student's *t-test* at $P \leq 0.05$ was applied to compare IC_{50} values of ABTS·+ and DPPH inhibition by quince leaf extract and BHT, respectively. Strength of relationship (r) between DPPH and ABTS·+ inhibition by leaf extract was estimated using Pearson product-moment correlation.

3 RESULTS AND DISCUSSION

3.1 LEAF EXTRACT CHARACTERISTICS

Results of chromatographic analysis of methanol leaf extract are represented in Table 1. A total number of nine compounds were detected under the experimental conditions applied to this analytical technique. Based on technical data provided for each peak, these nine compounds could be divided into two classes: four phenolic acids (C1, C7, C8, and C9) and five flavonoids (C2, C3, C4, C5, and C6). However, only six compounds were systematically identified as illustrated in Table 1. These compounds corresponded to five flavonoids (namely, quercetin-3-*O*-rutinoside, quercetin-3-*O*-galactoside, kaempferol-3-*O*-rutinoside, kaempferol-3-*O*-glycoside, and kaempferol-3-*O*-glucoside), and one phenolic acid (4-*O*-caffeoylquinic acid). Apparently, quercetin-3-*O*-rutinoside (rutin) constitutes the major phenolic compound in Tunisian quince leaves (largest peak). The same observation was also made by [3] who reported 36% of rutin in Tunisian quince fruit peels.

Literature describing the characterization of quince leaf extract is limited as compared to fruits for instance [23]. Oliveira et al. [8] reported that HPLC phenolic profile of Portuguese quince leaves revealed also the existence of nine compounds including those identified in this study. The rest of compounds matched exactly the three non- identified phenolic acids illustrated in Table 1: 3,5-*O*-dicaffeoylquinic acid, 3-*O*-caffeoylquinic acid and 5-*O*-caffeoylquinic acid. The latter was found to be the major compound (36.2%) followed by quercetin-3-*O*-rutinoside (21.1%) and kaempferol-3-*O*-rutinoside (12.5%).

Table 1. Chromatographic characterization of quince leaf methanol extract

Peak No.	Compound	Class	RT (min)	Parent ion (M-H)	UV spectrum (nm)
C1	4-*O*-caffeoylquinic acid	Phenolic acid	15.94	354.31	250 ; 324
C2	Quercetin-3-*O*-rutinoside (Rutin)	Flavonoid	30.99	610.53	256 ; 354
C3	Quercetin-3-*O*-galactoside (Hyperin)	Flavonoid	31.55	464.38	256 ; 354
C4	Kaempferol-3-*O*-rutinoside	Flavonoid	32.28	594.53	266 ; 350
C5	Kaempferol-3-*O*-glycoside	Flavonoid	33.22	448.38	266 ; 350
C6	Kaempferol-3-*O*-glucoside	Flavonoid	33.80	432	266 ; 320
C7	n.d	Phenolic acid	36.07	452	276 ; 320
C8	n.d	Phenolic acid	37.33	386	250 ; 312
C9	n.d	Phenolic acid	37.71	386	312

n.d: not determined

In fact, most of the published data on quince leaf characterization originate from Portugal where, unlike our study, 5-*O*-caffeoylquinic acid has been always observed as the major compound ranging from 10.7 mg/g [5] to 15.7 mg/g [12]. Oliveira et al. [8] showed that the geographical origin as well as the sampling season of leaves influenced their phenolic composition in Portugal. Within the same geographic area, Costa et al. [12] identified only six phenolic compounds in methanol extracts of quince leaves, three phenolic acids and three flavonoids. They attributed this lack of detection to low extraction effectiveness. In this study, the presence of five flavonoids in leaf methanol extracts (Table 1) is not surprising since this type of compounds act as UV filters, protecting some fragile cell structures, such as chloroplasts, from photooxidation [8], [12].

Quantification based on absorbance determination revealed that total phenolic and flavonoid contents were 235.66 mg GAE/g methanolic extract and 17.6 mg QE/g methanolic extract corresponding to 52.52 mg GAE/g dry leaves and 4 mg QE/g dry leaves, respectively (extraction yield = 22.3%). These values represent a respectable content that characterizes plants used for herbal medicine. For example, Sreelatha and Padma [24] pointed out that in *Moringa oleifera* mature leaves, total phenolic and flavonoid compounds were 46 and 2.7 mg/g, respectively. Oliveira et al. [8] already pointed out that a total phenolic mean content of 10.3 mg/g is considered as relatively high, qualifying quince leaves as a good and cheap source of bioactive constituents. Using 5-*O*-caffeoylquinic acid (CA) as calibration standard, it was reported that phenolic compounds in Portuguese quince leaves contained a mean of 220 mg CA/g dry leaves [12]. This was significantly two-fold higher than CA content in green tea. In their comparative study, Teleszko and Wojdyło [7] showed that the highest concentration of total polyphenols was observed for Polish quince leaves (89.6-175.4 mg/g dw) followed in a decreasing order by cranberry, apple, chokeberry, Japanese quince, bilberry and blackcurrant leaves.

As compared to the composition of quince edible parts (hole fruit, pulp, peel, or jam), it has been reported that the total phenolic content of leaves is higher owing to higher drying yields [7], [8], [25]. Accordingly, analytical results showed that Spanish quince peels have a total polyphenols content of 5.81 mg GAE/g, nine-fold lower than that determined in our leaves [6]. Comparable total content of phenolic compounds of 4.85 mg/g was also observed for quince jam [26]. In Tunisian quince fruits, Fattouch et al. [3] found that the total phenolic mean contents of the pulp and peel parts were 0.42 and 1.31 mg/g fw, respectively. Same content variation has been also reported for Indian quince pulp and peel (0.67 and 0.97 mg GAE/g fw, respectively) [27]. This confirms that quince leaves, based on lower water content and different physiological function, contain significantly higher concentration of phenolic compounds than fruits independently of the geographical origin and distribution.

3.2 ANTIOXIDANT CAPACITY

Antioxidant capacity assays may be broadly classified as single electron transfer- (SET) and hydrogen atom transfer- (HAT) based assays. SET assays are much easier and measure the capacity of an antioxidant in the reduction of an oxidant, which changes color when reduced [28]. DPPH free radical-scavenging activity has been largely used as a routine screening method for testing the antiradical potential of a large variety of compounds [29]. It is actually the most published antioxidant activity reported for different quince parts. In this study, both quince leaf extract and BHT presented a steep increasing concentration-dependent antiradical activity up to 120 µg/mL. This was reflected by comparable IC_{50} values at $P \leq 0.05$ (38.4 and 36.5 µg/mL, respectively) as shown in Fig. 3.

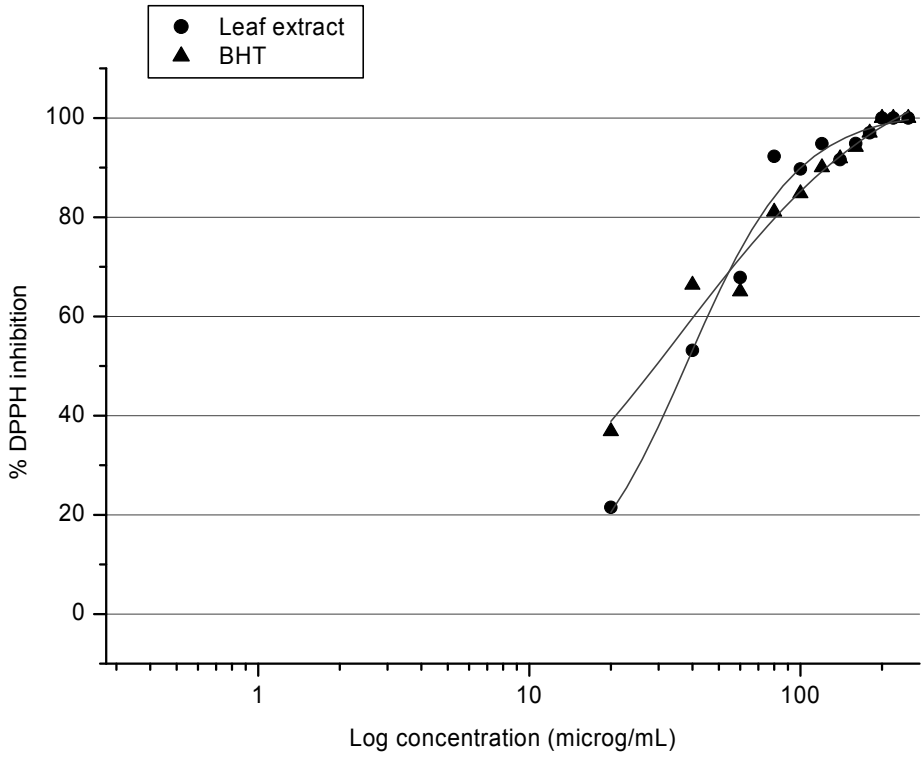

Fig. 3. Sigmoidal regression of DPPH (2,2-diphenyl-1-picrylhydrazyl) percent inhibition by quince leaf extract and BHT. Values are means of triplicate measurements. Half maximal 50% inhibitory concentrations (IC_{50}) of quince leaf extract and BHT were calculated using Origin 6.0 software.

Costa et al. [12] investigated DPPH free radical-scavenging capacity in twelve healthy quince leaf samples collected in various seasons and regions of Portugal. They reported IC_{50} values varying between 14.5 and 27.6 µg/mL with a mean average of 21.6 µg/mL. This is actually higher than the antiradical activity observed for Tunisian quince leaves but lower than green tea (12.7 µg/mL) and ascorbic acid (8.1 µg/mL), for instance [12]. For quince edible parts, pulp and peel extracts showed respectively IC_{50} values of 600 and 800 µg/mL for DPPH activity, while seed extract presented much lower antioxidant potential (12.2 mg/mL) [30]. This is nonetheless far lower than antiradical activity reported for leaf extract. We noted also comparable TEAC values for leaf extracts and BHT: 1.25 and 1.20 mmol TEAC/g dw, respectively. In their study on Tunisian quince fruits, Fattouch et al. [3] found higher TEAC values for peel than pulp: 4.27 against 3.33 g TEAC/100 g fw, respectively. As suggested, hydroxycinnamic acid derivatives seem to be the main driving force behind DPPH free radical-scavenging activity [2], [25]. The antioxidant effect of caffeoylquinic acids can be explained by the presence of a catechol group, which confers a great stability to phenoxyl radicals by participating in electron delocalization. Additionally, conjugated double bond in the side chain of a catechol group is likely to have great effect in stabilizing the putative phenoxyl radical and, therefore, in enhancing antiradical activity [2], [12], [25].

The inhibition of the pre-formed radical monocation of 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS·+) is illustrated in Fig. 4. As for DPPH activity, quince leaf extracts and BHT dose-dependently reduced ABTS·+ until 100% inhibition at 500 µg/mL. However, BHT effect was slightly stronger within this concentration range yielding an IC_{50} value of 128.2 µg/mL as compared to that of leaf extracts (161.4 µg/mL). In addition, there was no significant difference in their TEAC (0.73 and 0.87 mmol/g dw, respectively). Comparable values were also reported for Polish quince leaves (1.16 mmol TEAC/g dw), considered as the strongest among all studied plant species [7]. In contrast, Van Der Werf et al. [31] found lower ABTS·+ activity for French common quince fruit sugar-free polar extract (SFPE): 0.22 mmol TEAC/g SFPE.

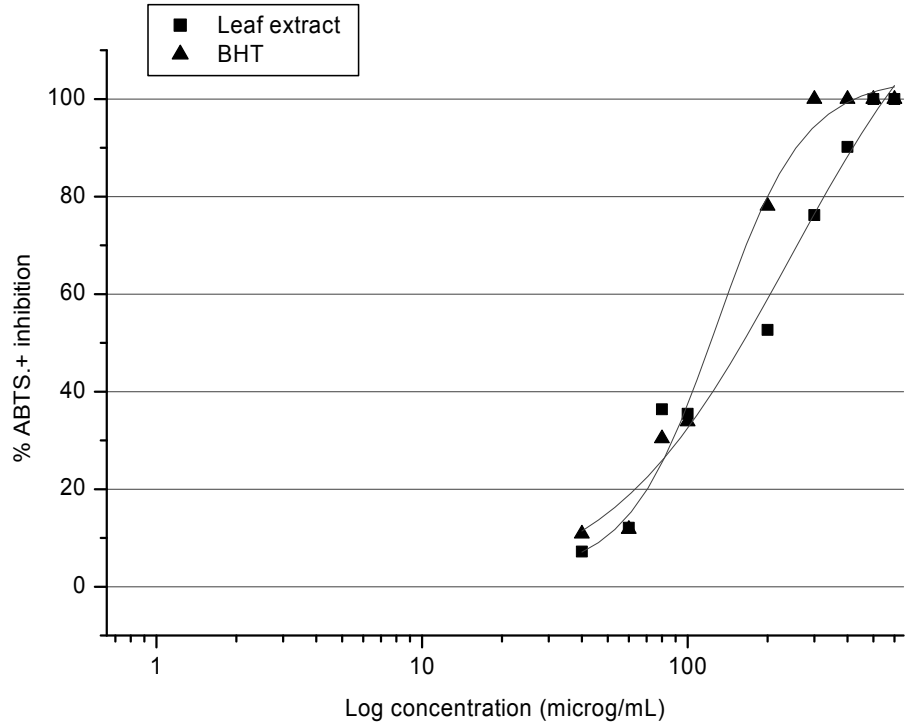

Fig. 4. Sigmoidal regression of ABTS˙+ (2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid) percent inhibition by quince leaf extract and BHT. Values are means of triplicate measurements. Half maximal 50% inhibitory concentrations (IC_{50}) of quince leaf extract and BHT were calculated using Origin 6.0 software

In their investigation on plant species of the genus *Chaenomelese*, close relative to quince, Du et al. [32] already qualified of strong ABTS·+ activities in fruits varying between 0.19 and 0.31 mmol Trolox/g fw. In other medicinal plants, Shi et al. [33] determined for instance a much lower TEAC value (0.89 µmol/g dw) in water extracts of the aromatic herb *Artemisia selengensis.* They found also a very strong correlation between ABTS·+ inhibition and total phenolic acid content. Finally, DPPH and ABTS·+ inhibition tests were very highly correlated in this study (r = 0.82) proving that only one test is sufficient to highlight the antioxidant potential of quince leaf extract.

4 CONCLUSION

Quince leaves constitute a promising natural source rich in bioactive compounds, namely caffeoylquinic acids, quercetin and kaempferol heterosides. Furthermore, leaves are low cost and are widely available after fruit harvest. The antioxidant and antiproliferative activities described for these materials may be indicative of the interest in quince leaves as a natural source of health promoting compounds, suitable for application in nutritional/pharmaceutical fields, in the prevention and treatment of free radical-mediated human chronic pathologies, such as cardiovascular diseases and cancer.

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